The host range of the mycoinsecticide *Beauveria bassiana* includes both the greater wax moth (*Galleria mellonella*) and the cabbage looper (*Trichoplusia ni*). Larvae of *G. mellonella* and *T. ni* were infected individually with five diverse strains of *B. bassiana* and mortality was followed over time. Mathematical analyses of these data indicated that the conventional parameter of LT₅₀ was the product of two strain-specific parameters that may be described as “onset” of mortality and subsequent “rate” of mortality. These parameters were compared with quantitative data on the production of enzymes from the same strains cultured *in vitro* on defined medium containing purified cuticle from either *G. mellonella* or *T. ni*. Levels of specific cuticle-degrading enzymes such as chitinase, chymoelastase, chymotrypsin, and esterase showed relationships with specific virulence parameters.

**KEY WORDS:** *Beauveria bassiana; Galleria mellonella; Trichoplusia ni; virulence; chitinase; chymoelastase; chymotrypsin; esterase.*

**INTRODUCTION**

The usefulness of entomopathogenic fungi has long been recognized (see references in Gillespie, 1988). The entomopathogenic fungus *Beauveria bassiana* infects a wide range of insects and has potential for development into a practical insect biocontrol agent (Bidochka and Khachatourians, 1987; McCoy, 1990). Because of their distinctive mode of infection, fungi may play a unique or complimentary role as insect biocontrol agents. Unlike bacterial and viral pathogens of insects, fungi need not be consumed by their hosts in order to be infective. Instead, fungal spores are able to directly breach the external insect cuticle (Pekrul and Grula, 1979). It has long been suspected that entomopathogenic fungi are enabled in this novel mode of infection by the production of cuticle-degrading enzymes (Samsonakova *et al.*, 1971; Smith *et al.*, 1981; Dean and Domnas, 1983; Charnley, 1984). It is reasonable to suppose that these enzymes are important, and perhaps determinative, in the effectiveness of infection. Despite this, little is known about the role of cuticle-degrading enzymes in determining total strain virulence.

This study is the continuation of our earlier work (Gupta *et al.* 1992) in which production and regulation of hydrolytic enzymes by *B. bassiana* during growth on defined media and insect cuticle was studied. This paper deals with the efficacy of five strains of *B. bassiana* from widely divergent sources and geographical sites, against two host larvae, *Galleria mellonella* and *Trichoplusia ni*. Laboratory bioassays were developed, and derived quantitative virulence parameters were compared with the production of specific cuticle-degrading enzymes.

**MATERIALS AND METHODS**

**Organisms**

All strains were from the ARS Culture Collection (Peoria, IL). Strains were selected to reflect divergent isolation sources and/or sites (Table 1). Spore stocks were prepared by harvesting sporulated spread plates of Sabouraud–maltose agar containing 1% (w/v) yeast extract.

**Virulence Bioassays**

Larvae of the greater wax moth (*G. mellonella*) weighing 0.15 to 0.20 g/larva were selected for bioassays. An equal number of spores (6 × 10⁷) were taken in a 400-μl vol of water and 10 larvae of *G. mellonella* were infected individually with five diverse strains of *B. bassiana* and mortality was followed over time.
TABLE 1
Strains of Beauveria bassiana Used in This Study

<table>
<thead>
<tr>
<th>NRRL No.</th>
<th>Source Collection Site</th>
<th>Source Collection Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>3108</td>
<td>European corn borer</td>
<td>Unknown</td>
</tr>
<tr>
<td>13050</td>
<td>Corn</td>
<td>Georgia, U.S.A.</td>
</tr>
<tr>
<td>20698</td>
<td>Dysdercus sp. (Hemiptera)</td>
<td>Peru</td>
</tr>
<tr>
<td>20699</td>
<td>Soil</td>
<td>Oregon, U.S.A.</td>
</tr>
<tr>
<td>20700</td>
<td>Japanese beetle</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Enzyme Assays

Enzyme assays were carried out on supernatants from liquid cultures of different strains of B. bassiana as described in our earlier paper (Gupta et al., 1992). Liquid cultures contained a basal salts medium (0.06% MgSO₄, 0.05% NaCl, 1.5% KH₂PO₄, 0.001% FeSO₄·7H₂O, 0.001% ZnSO₄) amended with 0.5% (w/v) insect cuticle. Cuticles from larvae of G. mellonella and T. ni were prepared by dissection according to the method of Hackman (1980). Dried cuticles were gas-sterilized using propylene oxide. Media containing cuticle were subjected to heating at 65°C for 15 min to inactivate any endogenous enzymes. Media were inoculated with spores to a concentration of 2 x 10⁷/ml. Cultures were incubated at 200 rpm at 26°C for 96 hr. After this period, the cultures were examined microscopically to rule out bacterial contamination and then centrifuged at 2000g for 10 min. Supernatants were collected and stored at -20°C for enzyme assays. Enzyme assays were performed on supernatants before and after sterilization by passage through 0.22-µm filters (Costar, Cambridge, MA). There was no measurable effect of sterilization on the enzyme level. In preliminary studies, we found no effect of dialyzing samples, so this step was omitted. Enzyme activities were operationally defined by their substrates, all obtained from Sigma Chemical Co. (St. Louis, MO), which were: trypsin, N-benzoyl-Phe-Val-Arg-p-nitroanilide; N-acetyl glucosaminidase (NAGase), p-nitrophenyl-N-acetyl-β-D-glucosaminide; and endochitinase, purified crustacean chitin. Total chymotrypsin activity was measured using N-carbobenzoxy-L-tyrosine-p-nitrophenyl ester as described by Walsh and Wilcox (1970). Presumptive chymoelastase activity was estimated using suc-Ala-Ala-Pro-p-nitroanilide as described by St. Leger et al. (1987). As reported earlier, we found that chymotrypsin and chymoelastase values from B. bassiana strains were not proportional under different nutritional regimes, suggesting that chymotrypsin and chymoelastase assays measured at least two distinct protease activities (Gupta et al., 1992).

Statistical Analyses

Full logarithmic plots of insect mortality against time (illustrated by Fig. 1) were analyzed by first-order linear regression equations (SigmaPlot version 4.1; Jandel Scientific, Corte Madera, CA). Correlation coefficients, slopes, and x-axis intercepts of regression lines were calculated by standard means. Enzyme values varied by up to 15% standard deviation of the mean among parallel cultures, and assays were reproducible to within 5% standard deviation of the mean (Gupta et al., 1992). Relative enzyme values were dipped in this solution and left on G. mellonella medium (North Carolina Biological Supply, NC). The mortality of larvae was recorded each day for 12 days. Parallel (untreated or treated with sterile distilled water) controls were included. Controls showed no mortality over the course of the experiments. Postmortem autopsies were performed to confirm that deaths of infected larvae were due to B. bassiana fungus. At the end of 12 days, 1 or 2 sample cadavers per infection were surface sterilized by immersion in 95% ethanol and repeated washing in sterile distilled water and dissected on a rich medium plate which included ampicillin at 100 µg/ml and cycloheximide at 1.0 µg/ml to prevent the growth of bacteria and nonentomogenous fungi. All mortality was associated with B. bassiana infections. Uninoculated control samples were also sacrificed, autopsied, and found to be free of fungal infection. Larvae of the cabbage looper (T. ni) were similarly tested, using modifications previously described (El-Sayed et al., 1989). Reported bioassay results were characteristic of sequentially repeated experiments.

FIG. 1. Pathogen-related mortality of Galleria mellonella as a function of time. Plot is full logarithmic, illustrating log of mortality as a function of log time. Symbols: ○, NRRL 3108; □, NRRL 20698; ■, NRRL 20699.

TABLE 2
B. bassiana Virulence against Galleria mellonella

<table>
<thead>
<tr>
<th>NRRL No.</th>
<th>Onset Rate of mortality</th>
<th>LT₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>3108</td>
<td>3.82</td>
<td>7.70</td>
</tr>
<tr>
<td>13050</td>
<td>3.37</td>
<td>7.16</td>
</tr>
<tr>
<td>20698</td>
<td>2.80</td>
<td>4.36</td>
</tr>
<tr>
<td>20699</td>
<td>1.64</td>
<td>4.20</td>
</tr>
<tr>
<td>20700</td>
<td>2.15</td>
<td>4.20</td>
</tr>
</tbody>
</table>
RESULTS

Direct plots of pathogen-related insect mortality vs time were found to be distinctly nonlinear (data not shown). Among various transformations of these data, we found that linear relationships were formed by full logarithmic plots of mortality against time (i.e., log of mortality as a function of log time), illustrated in Fig. 1. Linear regression lines for these functions exhibited correlation coefficients of greater than 0.8. As linear functions, these lines may be simply defined by their intercepts and slopes. As illustrated in Fig. 1, the x-axis intercept of a given regression line is a mathematical estimate of the time of death of the first larva in a test group, based on the kinetics of the entire population. Biologically, this value may be interpreted as “onset of mortality.” On the other hand, the slope of a regression line describes the average “rate of mortality” for a given fungal strain. As exemplified in Fig. 1, each strain produced a unique mortality function line. One conventional measure of pathogen virulence has been LT$_{50}$, the time required to kill 50% of the insect population. Apparent LT$_{50}$ values were thus shown to be the consequence of both onset of mortality and rate of mortality. Both onset and rate appeared to vary independently among different strains of $B. bassiana$.

Calculated virulence parameters for strains of $B. bassiana$ against $G. mellonella$ are summarized in Table 2. Strains NRRL 20699 and 20700 showed LT$_{50}$ values about half that of other strains tested. NRRL 20699 exhibited the earliest onset of mortality, but acted at a relatively slow rate. NRRL 20700, in contrast, had the highest rate of mortality and the second earliest onset of mortality, resulting in the lowest LT$_{50}$ of all five strains.

Virulence parameters of the same strains against $T. ni$ are given in Table 3. Overall, strains were somewhat more active against $T. ni$ (lower LT$_{50}$ values),

### TABLE 3

$B. bassiana$ Virulence against $Trichoplusia ni$

<table>
<thead>
<tr>
<th>NRRL No.</th>
<th>Onset (days)</th>
<th>Rate of mortality (log mortality/log time)</th>
<th>LT$_{50}$ (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3108</td>
<td>3.50</td>
<td>8.96</td>
<td>4.2</td>
</tr>
<tr>
<td>13050</td>
<td>3.82</td>
<td>4.95</td>
<td>5.3</td>
</tr>
<tr>
<td>20698</td>
<td>3.22</td>
<td>11.80</td>
<td>3.7</td>
</tr>
<tr>
<td>20699</td>
<td>1.76</td>
<td>4.12</td>
<td>2.6</td>
</tr>
<tr>
<td>20700</td>
<td>3.10</td>
<td>7.15</td>
<td>3.9</td>
</tr>
</tbody>
</table>

for $B. bassiana$ strains cultured on insect cuticle were directly plotted against mortality parameters determined for that host (Figs. 2–5). Simple spline lines were added to these figures to suggest relationship trends (SigmaPlot 4.1, omitting outlier values).

FIG. 2. Relationships of cuticle-degrading enzymes with $Beauveria bassiana$ onset of mortality against $G. mellonella$. A, endochitinase; B, chymoelastase; C, chymotrypsin.
resulting generally from greater rates of mortality. However, NRRL 20699 exhibited a distinctly early onset of mortality as well as the highest virulence of tested strains. Virulence parameters against T. ni did not parallel those found against G. mellonella. For example, NRRL 20698, which exhibited the lowest rate of mortality against G. mellonella, showed by far the highest rate of mortality against T. ni.

To test the potential role of cuticle-degrading enzymes in determining overall strain virulence, enzyme levels were compared with specific virulence parameters. Figures 2A–2C plot the relationship between the cuticle-degrading enzymes chymoelastase, chymotrypsin, and endochitinase and the virulence parameter onset of mortality against G. mellonella. As shown, higher levels of these enzymes appeared to be related to early onset of mortality. Other enzymes tested but not shown (trypsin, NAGase, and esterase) did not show a discernible trend. In contrast, the rate of mortality of B. bassiana strains against G. mellonella showed apparent relationships with the enzymes esterase and NAGase (Figs. 3A and 3B).

Virulence parameters against T. ni larvae appeared to be related to a different set enzymes. Onset of mortality showed relationships with chymoelastase and chymotrypsin (Fig. 4), and rate of mortality showed relationships with both NAGase and endochitinase (Fig. 5).

**DISCUSSION**

It is commonly understood that pathogen-related mortality of insects follows a logarithmic curve, and strain-specific differences in these functions have previously been reported for B. bassiana (Samsinakova and Kalalova, 1983). Despite this, LT50 continues to be conventionally used to describe strain virulence. We suggest that the parameters onset of mortality and rate of mortality, which completely define the logarithmic function, may be more useful in understanding the factors determining biologically relevant strain differences.

A number of factors undoubtedly operate in determining the virulence in an insect host-pathogen relationship. Fungal pathogenesis is a complex and multifactorial phenomenon, with particular virulence factors coming into play at various stages of infection and death. The production of cuticle-degrading enzymes...
has long been proposed to be a prerequisite for fungal infection (Samsinakova et al., 1971; Smith et al., 1981; Dean and Domnas, 1983; Charnley, 1984), and some studies have suggested that they may be major determinants of virulence (e.g., Paris and Ferron, 1979; St. Leger et al., 1988; Silva and Messias, 1986).

The current study supports the notion that cuticle-degrading enzymes may determine not only specific virulence parameters, but also host specificity. It is known that the composition of insect cuticle varies from species to species (Hackman, 1974), and that regulation of degradative enzymes responds to these differences (Gupta et al., 1992). Even if enzymes are not causative, enzyme–virulence relationships could prove useful in developing enzyme-based screening methods to identify new fungal isolates with desired virulence characteristics. Rational screening programs could offer new tools for biological control of agricultural pests. If a direct role for cuticle-degrading enzymes is established, genetic studies could promote the development of strains improved for specific applications.

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REFERENCES


